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High Throughput Screening for Cyanovirin-N Mimetics Binding to HIV-1 gp41

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ABSTRACT

The human immunodeficiency virus type-1 (HIV-1) envelope glycoprotein gp41 is an important mediator of viral entry into host cells. Previous studies showed that the virucidal protein cyanovirin-N (CV-N) bound to both gp120 and gp41, and that this binding was associated with its antiviral activity. We constructed an HTS assay based on the interaction of europium-labeled CV-N with recombinant glycosylated gp41 ectodomain to support identification of small-molecule mimetics of CV-N that might be developed as antiviral drug leads. Primary screening of over 107,000 natural product extracts in the assay yielded 347 confirmed hits. Secondary assays eliminated extracts that bound directly to labeled CV-N or for which the simple sugars mannose and *N*-acetylglucosamine blocked the interaction with gp41 (lectin activity). Extracts were further prioritized based on anti-HIV activity and other biological, biochemical, and chemical criteria. The distribution of source organism taxonomy of active extracts was analyzed, as was the cross-correlation of activity between the CV-N–gp41 binding competition assay and the previously reported CV-N–gp120 binding competition assay. A limited set of extracts was selected for bioassay-guided fractionation.

INTRODUCTION

THE ROLE THAT THE human immunodeficiency virus (HIV) envelope glycoprotein gp41 is presumed to play in virus–cell fusion makes it an attractive drug target.[1,2] Agents that bind to gp41 may be capable of interfering with virus–cell fusion and thereby inhibiting HIV infection.[3] For example, T-20, a synthetic 36-amino-acid peptide that binds to crucial conformational elements of gp41, has recently shown efficacy in clinical trials.[4,5] Interference with the viral envelope glycoproteins (gps) that mediate virus–cell fusion may provide a broadly applicable model for discovering antiviral drugs.[6,7]

Cyanovirin-N (CV-N) is an 11-kDa protein originally isolated from the cyanobacterium *Nostoc ellipsosporum*, and subsequently produced recombinantly in *Escherichia coli*.[8,9] CV-N potently inactivates a broad spectrum of HIV strains, including T-tropic, M-tropic, dual tropic, primary isolates, and related immunodeficiency viruses.[8] Given its lack of toxicity and high resistance to physicochemical denaturation, CV-N is a promising new anti-HIV substance for both potential therapeutic and prophylactic applications.[8,10,11]

Although the precise antiviral mechanism of CV-N has not been fully defined, recent studies have shown that the antiviral activity of CV-N is associated with its high-affinity binding to the HIV envelope glycoproteins gp120 and gp41.[8,12] The binding of CV-N appears to occur in an oligosaccharide-specific manner to *N*-linked, high-mannose glycans present on the glycoproteins.[13] The interaction of CV-N with gp41 was 2 logs weaker than that with gp120, and the stoichiometry differed, with gp41 being bound in an approximately equimolar ratio, whereas gp120 was bound in a ratio of about 5:1 (CV-N:gp120).[13,14]

Previously, we have reported on an HTS assay based on gp120–CV-N interactions.[15] The results of that assay indicated that the binding interaction between gp120 and CV-N was so strong, and the stoichiometry so high, that very few lead extracts were identified. After recent reports indicated that CV-N interacted with both gp120 and gp41 through similar polar and electrostatic interactions involving the same high-mannose oligosaccharides (Man-8 and Man-9), it was decided that a more fruitful approach to screening for CV-N mimetics might be to use gp41 as a target (1:1 stoichiometry, K_d = 606 nM) rather than gp120 (5:1 stoichiometry, K_d = 2–35 nM).

The goal of this study was to design an HTS assay, based on

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the interaction of fluorescently labeled CV-N with the recombinant ectodomain of gp41, that would be capable of detecting compounds in natural product extracts that interfere with the binding of labeled CV-N to gp41. Such potential "mimetic" molecules that might bind to gp41 in a region similar to that of CV-N might likewise block HIV infection and provide new antiviral leads from different structural classes. The assay strategy was based on our recent experience with a CV-N–gp120 competition binding assay.[15] One goal was to determine if the stoichiometric binding interaction of CV-N with gp41 might provide a more productive screening target than the interaction of CV-N with gp120.

METHODS AND MATERIALS

A solid-phase assay with time-resolved fluorescence detection was designed analogous to the previously reported CV-N–gp120 competition binding assay[15] using a Quadra96® (Tomtec, Hamden, CT) for assay setup, a MultiPROBE® II (Packard Instrument Company, Meriden, CT) for dilutions and control additions, and a Victor^{2}™ (Perkin Elmer Life Sciences, Boston, MA) to read the plates in time-resolved mode.

Label

CV-N was labeled (1.2–1.8 label molecules per protein molecule) with a europium chelate by Perkin Elmer Life Sciences (Akron, OH). The labeled CV-N had anti-HIV activity[16] equivalent to unlabeled CV-N (data not shown). The binding of labeled CV-N to gp41 in an enzyme-linked immunosorbent assay format was essentially identical to that for unlabeled CV-N (data not shown).

HIV-1 gp41

The purified glycosylated ectodomain of HIV-1 gp41 (amino acids 546–682) produced in *Pichia pastoris* was purchased from Viral Therapeutics, Inc. (Ithaca, NY). Aliquots of 25–50 ng/well were bound to MaxiSorp™ 96-well plates (Nalge Nunc International, Naperville, IL) in a volume of 100 μl Dulbecco's phosphate-buffered saline (DPBS). Plates were washed with DPBS with 0.1% Triton X-100 and blocked with 1% bovine serum albumin (BSA) in DPBS. The assay was run in the presence of the blocking buffer.

Test samples

Plated extracts and pure compounds were obtained from the National Cancer Institute (NCI) repository (dtp.nci.nih.gov/repositories.html). Pure compounds were provided as 10 mM solutions in dimethylsulfoxide (DMSO), while natural product extracts were provided as 50 μg dry extract per well. An extract challenge plate containing a variety of nuisance compounds and commonly bioactive metabolites was used to investigate the robustness of the assay to crude extracts. In addition, pure compound sets consisting of pure natural products (4174 compounds) and a structural diversity set of synthetic compounds (1990 compounds), provided by the NCI Developmental Therapeutics Program (dtp.nci.nih.gov), were screened. Compounds and extracts were dissolved in DMSO, 50% DMSO, or distilled water depending on solubility class. DMSO was found to have no effect on binding at concentrations of less than 10% of the final assay volume.

Assay protocol

Samples were preincubated for 1 h at room temperature with bound gp41. To generate a set of dose–response controls, 25 μl of unlabeled CV-N from stock solutions of 400, 200, 100, and 50 ng/ml was added simultaneously to four control wells. Eu^{+3}-labeled CV-N was then added in 25-μl aliquots to all wells (final concentration 20 ng/well) and the plates were incubated for 1 h at room temperature. The plates were washed 3 times with phosphate-buffered saline containing 0.05% Tween 20. A total of 100 μl of DELFIA® enhancer solution (Perkin Elmer Life Sciences) was added to each of the wells to solubilize the bound europium, and europium fluorescence was measured in time-resolved mode with a time delay of 400 μs, using standard instrument parameters for europium time-resolved fluorescence.

Criteria for activity

Displacement of more than 60% of label at 100 μg/ml final concentration for extracts or 10 μM final concentration for pure compounds was considered a positive result. An immediate retest of plates in which wells were observed to be active was used for confirmation.

Dereplication and prioritization tools

The criteria that we selected to define an ideal extract for further fractionation included the following: the extract constituents would not bind directly to labeled CV-N; binding to gp41 would not be blocked by simple sugars; the activity would most likely be due to a low-molecular-weight compound; and the extract would be active in anti-HIV assays. Therefore, to prioritize confirmed active extracts, we conducted a series of experiments detailed in the following sections.

Washout experiment. A simple "washout" variation of the primary screening assay in which a wash step was inserted prior to addition of the labeled CV-N proved useful in detecting extracts that contained compounds that bound directly to labeled CV-N. The sample in the well was replaced with blocking solution (150 μl of 1% BSA in DPBS) before labeled CV-N was added. In a duplicate single-dose version of the experiment, reduction of binding of greater than 30% compared to unwashed parallel tests was taken to indicate binding to label. The experiment was also run in dose–response format for extracts in which the result of single-point experiments was inconclusive. It is conceivable that constituents with specific but very weak gp41 binding interactions could be eliminated by this procedure; however, such losses were considered acceptable in view of the efficient elimination of extracts that interacted directly with labeled CV-N.

Monosaccharide displacement experiments. The solid-phase binding assay was run in the presence of 0.1 M concentrations of the simple sugars mannose, *N*-acetylglucosamine (glcNAc), and xylose (as control) and compared to assays in the absence

of sugars. The majority of extracts were examined in a duplicate single-dose format in which the criterion for elimination was a reduction in binding of more than 30% caused by either mannose, glcNAc, or both. Inconclusive samples were repeated in the duplicate dose–response format shown in Figure 1.

Anti-HIV cytopathicity assay. The ability of extracts to inhibit the cytopathic effects of HIV-1 was studied as previously reported.[16]

Ultrafiltration. Aliquots of 0.25 ml of a 10-mg/ml solution of each extract in water were filtered using an Amicon® YM-10 Microcon® centrifugal filtration device (Millipore Corporation, Bedford, MA). The filtrate and retentate obtained were made up to equal volume and tested in a duplicate dose–response assay format in the CV-N–gp41 binding assay.

Polyamide solid-phase extraction. Aliquots of 10 mg of extract were dissolved in a minimum volume of water (0.5 ml or less) and loaded onto preequilibrated solid-phase extraction (SPE) tubes filled with 1.0 g of Macherey-Nagel MN-6 polyamide beads (Macherey-Nagel Inc., Easton, PA). Two column volumes of water were collected, then the receiver was changed and two column volumes of methanol were used for elution. Fractions were evaporated under nitrogen and lyophilized. Parent and daughter fractions were tested in duplicate dose–response format in the CV-N–gp41 binding assay, and relative potencies were compared.

Wide-pore C-4 SPE. Aliquots of 10 mg of extract were dissolved in a minimum volume of water and loaded onto pre-equilibrated 6-ml SPE columns filled with 500 mg of wide-pore C-4 bonded phase media (JT Baker, Phillipsburg, NJ). The columns were eluted with two column volumes in succession of water, methanol–water (1:2, v/v), methanol–water (2:1), and methanol. The four fractions were evaporated under nitrogen and lyophilized. Samples were tested in duplicate dose–response format.

RESULTS

Fourteen pure natural product compounds were active at a concentration of 10 μM (Table 1); however, these were all high-molecular-weight yeast mannans and related polysaccharides that directly bound the labeled CV-N, as shown by experiments in which the test substance was washed out of the well before labeled CV-N was added. Specifically, mannans from *Hansenula holstii* (NSC#174479 and NSC#174481), *H. capsulata* (NSC#174478), *H. minuta* (NSC#174473), *Pichia pinus* (NSC#174477) and *Rhodosporidium sphaerocarpum* (NSC#307194) displaced labeled CV-N from gp41 by 65%–98% at 10 μM. We previously found and characterized fungal mannans with similar properties in our CV-N–gp120 screening campaign.[15] None of the pure synthetic compounds in the NCI structural diversity set showed activity in the screening assay.

A total of 347 extracts of the 107,712 tested were confirmed to inhibit CV-N–gp41 binding. The source taxonomic distribution is given in Table 2. Aqueous extracts constituted 99% of active samples. The active extracts were first evaluated for their direct binding to labeled CV-N by a washout experiment in which the test sample was removed prior to addition of labeled CV-N. Approximately two thirds of the initial extracts selected (239/347) failed this test.

Incubation of the samples with either mannose or *N*-acetylglucosamine (0.1 M), the two monosaccharide units present in the high-mannose oligosaccharides of recombinant gp41, reversed inhibition of CV-N binding for a majority (77/108) of the remaining extracts. Two examples are shown in Figure 1, in which the addition of sugars markedly shifted the dose–re-

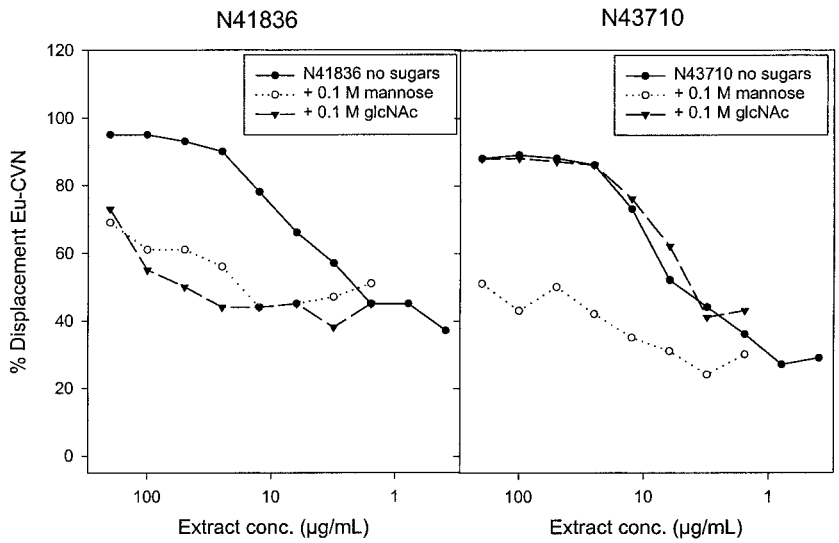

FIG. 1. Effect of addition of simple sugars on inhibition of binding of CV-N and gp41 by two plant extracts. N41836 is the aqueous extract of *Urera elata* (Urticaceae) wood, showing reversal of inhibition of both mannose and glcNAc, while N43710 is the aqueous extract of *Phaius tankervillii* (Orchidaceae), showing reversal of inhibition by mannose but not glcNAc.

Table 1. Natural Products Identified as Inhibitors of CV-N–gp41 Binding

NSC#	Percent displacement CV-N at 10 μM	Compound description	Compound source
100287	81	Antibiotic from *Paecilomyces* sp.	B. T. Sokoloff, Florida Southern College
174465	74	Antibiotic 9671	A. Ciegler, USDA
174473	65	*Hansenula minuta* phosphomannan	A. Ciegler, USDA
174477	69	*Pichia pinus* mannan	E. H. Pryde, USDA
174478	98	*Hansenula capulata* mannan	E. H. Pryde, USDA
174479	87	*Hansenula holstii* mannan	E. H. Pryde, USDA
174480	97	Antibiotic 9688	E. H. Pryde, USDA
174481	89	*Hansenula holstii* mannan	E. H. Pryde, USDA
246149	69	AT50	H. Ito, Mie University
285117	64	Black yeast from mountain laurel leaf	W. J. Nickerson, Rutgers University
286162	70	Black yeast strain from holly leaf	W. J. Nickerson, Rutgers University
294732	62	Polysaccharide from paint mildew strain	W. J. Nickerson, Rutgers University
307194	77	*Rhodosporidium sphaerocarpum* mannans	N. Otake, University of Tokyo
329703	78	Cell wall lytic enzyme	K. Iwai, Kyoto University

USDA, U.S. Department of Agriculture.

sponse curve. In one case, only mannose showed such an effect, whereas, in the second case, both mannose and glcNAc produced a shift. Evidence that the observed activity could be due to proteins in these samples was obtained by precipitation of a protein fraction with ammonium sulfate and by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, with HIV cytopathicity testing of the protein fraction. Thus these extracts were considered to contain lectins, and were not considered for further fractionation. The overall flow of dereplication and prioritization is illustrated graphically in Figure 2.

Among the lectin-containing extracts was one that had previously been shown in our laboratory to contain the *Myrianthus holstii* lectin, based on its activity in the HIV cytopathicity assay.[17] In general, the lectin-containing extracts appear to include representatives of three of the four major types of plant lectins, including monocot mannose-specific lectins, legume lectins, and chitin-binding lectins.[18] *Myrianthus holstii* lectin is an example of the latter class.

The 29 extracts that survived the dereplication process (i.e., were neither CV-N binders nor lectin like) were further prioritized for fractionation using ultrafiltration and SPE on both polyamide and wide-pore C-4 columns. These protocols were designed to segregate extracts, respectively, by molecular weight, polyphenolic content, and polarity. All of the extracts showed partial or complete retention of activity by a 10-kDa ultrafiltration membrane, indicating that the active species were likely to be of relatively high molecular weight. However, these data did not distinguish any extracts as clearly containing active small molecules. Use of polyamide columns to remove tannins had a negligible effect on activity of the extracts, indicating that tannins were not likely to be the source of the bioactivity. Use of wide-pore C-4 SPE of the extracts commonly yielded the most potent activity in the 33% MeOH fraction, although several extracts gave the best recovery of activity from the water eluate. The selected extracts were also tested in an anti-HIV assay,[16] and extracts possessing such activity

Table 2. Confirmed Hits/Extract Samples Screened (Percent Hits) by Taxonomy of Source Organism

Source organism taxonomy	Hits/extracts (%)
Marine Invertebrates	**76/13,552 (0.56%)**
Cnidaria	1/3070 (0.03%)
Crustacea	2/360 (0.5%)
Echinoderms	32/1347 (2.4%)
Mollusca	2/1518 (0.1%)
Sponges	19/10,148 (0.19%)
Tunicates	18/1798 (1.0%)
Higher Plants	**269/87,472 (0.31%)**
Annonaceae	13/2118 (0.6%)
Arecaceae	16/863 (1.9%)
Euphorbiaceae	14/4514 (0.3%)
Fabaceae	25/7286 (0.3%)
Moraceae	26/2024 (1.3%)
Olacaceae	23/368 (6.2%)
Solanaceae	8/866 (0.9%)
Urticaceae	12/500 (2.4%)
Other families	132/68,933 (0.2%)
Microbes	**1/4224 (0.02%)**
Algae	**1/2464 (0.04%)**
Total	**347/107,712 (0.32%)**

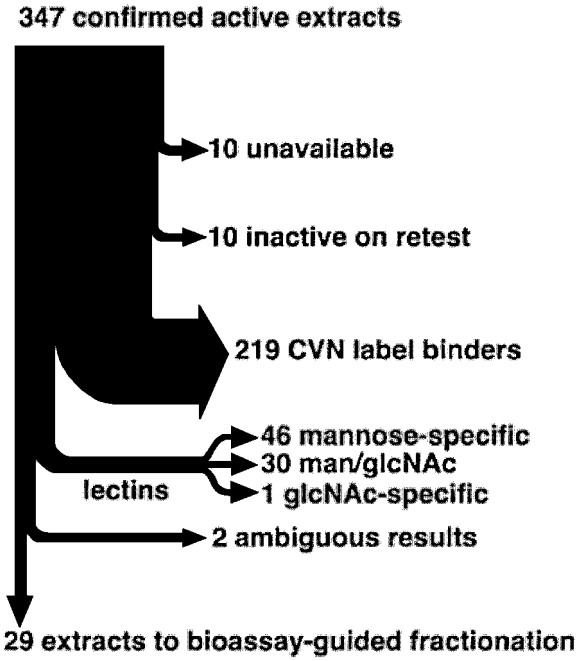

FIG. 2. Dereplication and prioritization statistics.

were retained while those without, or with marginal, anti-HIV activity were dropped from further consideration.

DISCUSSION

The goal of this screening campaign was to identify natural product extracts potentially containing low-molecular-weight, nonpeptidic compounds that bound the viral protein gp41 in a manner similar to CV-N. Such compounds are most likely to occur in the organic solvent extracts; however, only four such extracts out of over 50,000 tested inhibited binding of CV-N to gp41. This is similar to the rate observed with our CV-N–gp120 screening campaign. Furthermore, all of these showed substantially reduced inhibition of binding in a washout experiment, indicating that they bound to CV-N, not gp41. In contrast, for the aqueous extracts, we found a hit rate of approximately 0.5%. Of these, the majority ($n = 219$) were determined to bind CV-N; however, the rest appeared to bind to gp41. The remaining 108 extracts were sorted based on the ability of the simple sugars mannose or glcNAc to inhibit the binding. The majority of these extracts ($n = 77$) showed diminished binding in the presence of the two sugars, leaving an enriched set of 29 extracts for fractionation.

With several exceptions, the selected active extracts were derived from higher plants. Some of the 29 extracts were different plant parts of the same collection, or from different species from the same genus, which made it possible to group the extracts into a total of 15 distinct projects. Most plant parts (e.g., wood, roots, bark, leaves) were represented in this total, although no seed or fruit extracts were present in this group. The families Moraceae, Solanaceae, and Urticaceae accounted for 20 of the extracts. These families are recognized sources of lectins,[18] and whether these extracts contain novel lectins, or other substances that recognize oligosaccharide moieties with high specificity as does CV-N, will be of interest. Alternatively, the extracts may contain polar small molecules that are capable of minimizing CV-N binding to gp41. The results of fractionation studies will be reported in due course.

The historical database of activity in the previous gp120–CV-N screening campaign[15] was cross-correlated with the present results. In the previous screen, we concentrated on organic extracts, so only a limited number of aqueous plant extracts were tested; therefore, all but three of the extracts identified in the gp41 screening assay were not tested in the gp120 assay. All of the selected extracts displayed a level of activity in the gp120 interaction assay comparable to that found in the gp41 assay (i.e., >60% displacement of label at 100 μg/ml). This is to be expected because the glycosylation patterns of gp120 and gp41 are similar, and further highlights the importance of glycosylation in mediating the gp41 and gp120 binding of cyanovirin.

CV-N binds with high specificity to the mannose-containing oligosaccharides Man-8 and Man-9, on gp120 and gp41, and CV-N's antiviral activity appears to depend critically on the presence of these oligosaccharides.[13,14] CV-N is thus not directly comparable to lectins, which are proteins that bind monosaccharides with some specificity but with relatively weak affinity. Whereas most lectins offer at best micromolar binding affinity constants for monosaccharides, CV-N binds to gp41 and gp120 with nanomolar affinity.[14] Similarly, lectins with anti-HIV activity are typically active only at micromolar concentrations.

The primary "hit rate" of 0.3% that we realized in this assay is generally considered acceptable for the high-throughput screening of libraries. This rate is slightly higher than that found when we used gp120 as the target. When the extracts that appeared to bind labeled CV-N were eliminated, this rate dropped to approximately 0.1%. Further reduction in numbers (to 0.03%) was achieved by eliminating extracts where activity was blocked by mannose or glcNAc. Thus a manageable set of extracts could be identified and prioritized for fractionation in keeping with the capacity for bioassay and natural products chemistry of our laboratory.

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